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Development and characterization of a novel liposome-based formulation of SN-38

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Abstract

SN-38, 7-ethyl-10-hydroxycamptothecin, is the active metabolite of Irinotecan (CPT-11), a topoisomerase I inhibitor commercially available as Camptosar®. SN-38 is approximately 200–2000-fold more cytotoxic than CPT-11. Despite its promising anticancer potential, SN-38 thus far has not been used as an anticancer drug due to its poor solubility in any pharmaceutically acceptable solvents. In addition, SN-38 has low affinity to lipid membranes; it tends to precipitate in aqueous phase resulting in a very low drug-to-liposome entrapment. SN-38 also reversibly converts to an inactive open lactone ring structure at physiological pH. We have developed a novel, liposome-based SN-38 formulation (LE-SN-38). The formulation contains liposomes of uniform size distribution (<200 nm), and it is easy-to-use. Drug entrapment efficiency of the formulation is >95%. Long-term stability studies indicate that the lyophilized LE-SN-38 is physically and chemically stable for at least 6 months at 2–8 °C. In preclinical studies, LE-SN38 has shown promising results in terms of increased cytotoxicity against various tumor cell lines and better therapeutic efficacy towards xenograft mouse models compared to CPT-11.

Keywords: Liposomes; SN-38; CPT-11; Stability; Lyophilization; Anti-tumor activity

1. Introduction

SN-38, 7-ethyl-10-hydroxycamptothecin is an active metabolite of Irinotecan (CPT-11), a derivative of camptothecin. Current research suggests that the cytotoxicity of SN-38 is due to double-strand DNA damage produced during DNA synthesis when replication enzymes interact with the ternary complex formed by topoisomerase I, DNA and SN-38. Mammalian cells cannot efficiently repair these double-strand breaks (Physician Desk Reference, 2003a). CPT-11 was approved in the United States in 1996 and is commercially available as Camptosar® (Pharmacia Corp., Peapack, NJ) for use in the treatment of recurrent metastatic colorectal cancer. The metabolic conversion of CPT-11 to the active SN-38 occurs in the liver via carboxylesterase-mediated cleavage of the carbamate bond between the camptothecin moiety and a dipiperidino side chain (Tsuji et al., 1991; Satoh et al., 1994; Ahmed et al., 1999). Subsequently, SN-38 undergoes conjugation reactions to form the inactive glucuronide metabolite (Kono and Hara, 1991). The metabolic pathways of Irinotecan (CPT-11) are shown in Fig. 1. It is reported that only a small fraction (2–8%) of CPT-11 is ultimately converted to the active form of SN-38 (Rothenberg

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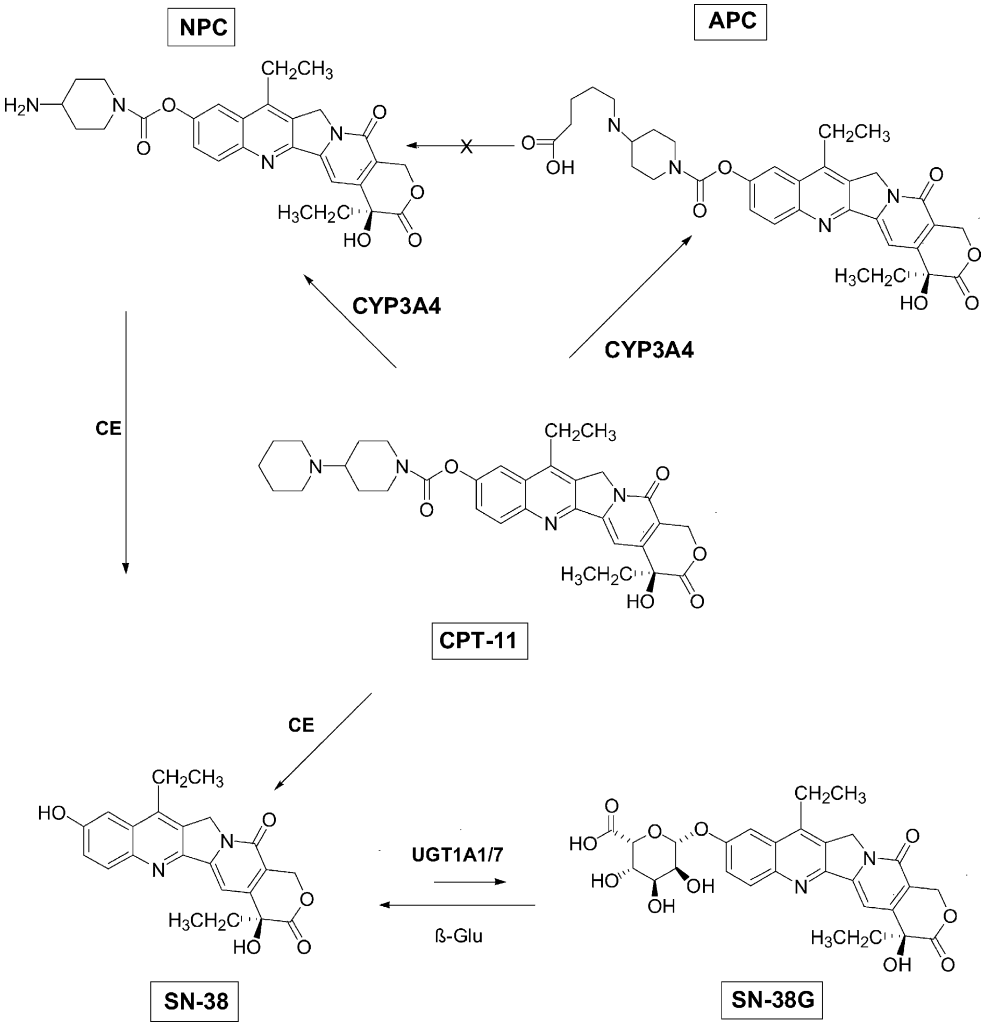

Fig. 1. Metabolic pathways of CPT-11 and SN-38. NPC: 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin; APC: 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino]-carbonyloxycamptothecin; CE: carboxylesterase; CYP3AX: cytochrome P-450 isoform 3A; UGT: UDP glucuronosyltransferase (Reference: Mathijssen, R.H.J. et al., 2001).

et al., 1993, 2001; Physician Desk Reference, 2003a). In addition, the conversion of CPT-11 (micromolar plasma levels) to SN-38 (nanomolar plasma levels) is highly variable and interpatient variability in the metabolism of SN-38 to form SN-38 glucuronide has been reported (Ohe et al., 1992; Gupta et al., 1994). The variability and unpredictability in the CPT-11 to SN-38 metabolic conversion rates pose significant life threatening toxicity risks and complicate clinical management of patients. SN-38, on the other hand, has an advantage over its camptothecin precursors in that it does not require activation by the liver, thereby eliminating the interpatient variability. Additionally, SN-38 is approximately 1000-fold more potent than CPT-11 as a topoisomerase I inhibitor purified from human and rodent tumor cell lines (Physician Desk Reference, 2003a). In vitro cytotoxicity studies suggest that SN-38 is up to 1000-fold more potent than CPT-11 against several tumor cell lines (Takimoto and Arbuck, 2001; Physician Desk Reference, 2003a). Moreover, the biological half-life of SN-38 is much longer than that of topotecan and CPT-11, thereby

representing a great pharmaceutical advantage over CPT-11 as a potentially highly effective antineoplastic agent. Nonetheless, SN-38 is poorly soluble in aqueous solutions, and is practically insoluble in most physiologically compatible and pharmaceutically acceptable solvents, including ethanol, polysorbate 80 and cremophor. Formulation of SN-38 in concentrated pharmaceutical delivery systems for parenteral administration is thus very difficult. In addition, SN-38 has a low affinity to lipid membranes and tends to precipitate into aqueous phase resulting in a very low drug-to-liposome entrapment (Wadkins et al., 1999). This low lipid affinity behavior of SN-38 has made the development of a liposome-based SN-38 formulation more challenging. Furthermore, SN-38 exists in two distinguishable forms, an active α-hydroxy-δ-lactone ring and an inactive carboxylate form at different pH conditions (Fig. 2). An acidic pH promotes the formation of the active lactone ring, while a more basic pH favors the inactive carboxylate formation. Crow and Crothers (1992) and Wani et al. (1987) indicated that a closed hydroxylactone ring moiety is an important structural requirement for successful drug interaction with the Topo I target and anti-tumor potency. Rapidly opening the lactone ring of camptothecins and their derivatives would result in the complete loss of biological activity (Wani et al., 1987). Unfortunately, it was reported that in PBS at 37 °C, the lactone ring of free camptothecin was rapidly hydrolyzed to form inactive carboxylate form with $t_{1/2}$ of 16.6 min (Burke et al., 1992). Burke and Mi and Mi and Burke confirmed that the lactone ring of almost all camptothecins opens rapidly in human plasma at pH 7.4 and 37 °C to form the inactive carboxylate form. The $t_{1/2}$ of this conversion was about 12 min, and within 2 h, 99% of the active drug was converted to the inactive carboxylate form (Burke and Mi, 1993; Mi and Burke, 1994).

It has been shown that liposome delivery systems can enhance drug solubility, reduce toxicity associated with free anticancer drugs (Gregoriadis, 1991; Langer, 1998) and improve stability of the drug by protecting the compound from chemical degradation or transformation (Burke et al., 1992; Mi and Burke, 1994). Several liposomal products have been proven to be more effective, less toxic and exhibit improved

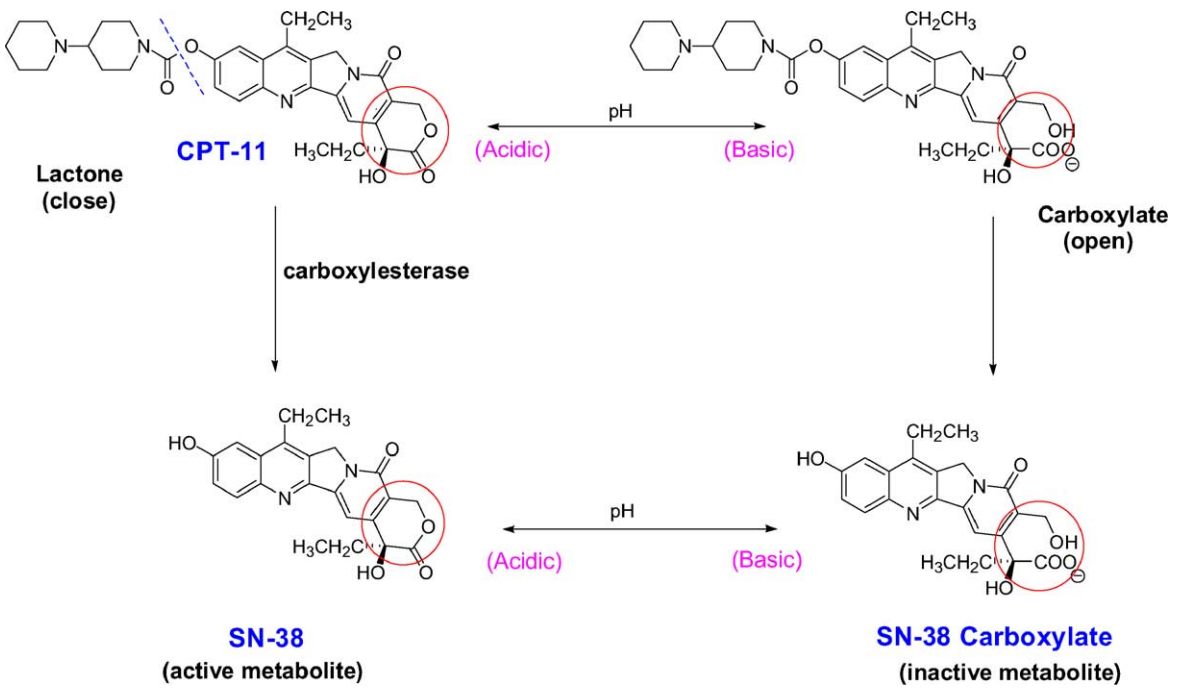

Fig. 2. pH-dependent equilibrium of CPT-11 and SN-38.

pharmacokinetic and pharmacodynamic profiles than free drugs (Physician Desk Reference, 2003b); however, the number of commercially available liposomal products is still limited (Zhang and Pawelchak, 2000). To be useful as a pharmaceutical product, the liposomal formulation should have a high drug to lipid ratio in order to reduce unnecessary lipid load to the patient, a relatively high drug entrapment to lower free drug in the product, a reasonably long shelf-life and a scalable manufacturing process.

The objectives of this study were (1) to develop a pharmaceutically acceptable formulation, (2) to fully characterize the formulation, (3) to evaluate the short- and long-term stability of the formulation, and (4) to evaluate the therapeutic efficacy of the formulation against various tumor xenogaft models.

2. Materials and methods

SN-38 was received from Qventas, Inc. (Newark, DE, USA). Camptosar®, Irinotecan hydrochloride injection (CPT-11) was purchased from Pharmacia (Kalamazoo, MI, USA). Purified phosphatidylcholine (DOPC), cardiolipin and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Methylene chloride, sodium chloride, USP grade, potassium phosphate, sodium phosphate, sodium hydroxide, NF grade, hydrochloric acid and chloroform (Ominsolv) were purchased from EM Science (EM Science, Gibbstown, NJ, USA). Sucrose, NF grade was obtained from Mallickrodt (Baker Inc., Phillipsburg, PA, USA). Dimethyl sulfoxide (DMSO) was received from Aldrich Chemicals (Milwaukee, WI, USA). Sodium lactate was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Finally, nitrogen, NF grade was obtained from BOC Gases (Carol Stream, IL, USA). All chemicals were used as received.

2.1. SN-38 and lipid assays

The HPLC system used for drug and lipid content analysis consisted of Agilent 1100 modules (Wilmington, DE, USA), a quaternary pump, mobile phase degasser, auto-sampler with thermostat and a column heater compartment. Agilent software, ChemStation, was used for data acquisition and analysis. For SN-38 drug content assay, a UV variable detector at a wavelength of 265 nm and an Agilent Zorbax SB-C18 column (4.6 mm × 250 mm, 5 μm) were utilized. The mobile phase consisted of a 50:50 (v/v) mixture of 25 mM NaH_2PO_4 (pH = 3.1) buffer and acetonitrile. The pH of the mobile phase was maintained at 3.1 to ensure the analyte, SN-38 was in the closed lactone ring form during the assay except for pH equilibrium study where the pH of the mobile phase was adjusted to different pH conditions.

During the assay, an aliquot of 20 μl of sample was injected in duplicate into the HPLC system at a flow rate of 1 ml/min. The SN-38 content in the formulation was quantitatively determined using external standards (Xuan et al., 2003). For lipid analysis, an evaporative light scattering detector (Polymer Laboratories or Alltech ELSD, model 2000) and a diol column (equilibrated at 40 °C, 250 mm × 4.6 mm, 5 μm, ASTEC, NJ, USA) were used. The mobile phase consisted of a 71:26:3 (v/v/v) mixture of chloroform:methanol:ammonium acetate (0.01 M NH_4AC, pH 9) at a flow rate of 1.0 ml/min. Lipids were quantitatively determined using a standard curve comprised of a minimum of six working standard concentrations (Simonzadeh et al., 2003).

2.2. Solubility studies

Briefly, excess amounts of SN-38 were added to the screw capped scintillation vials containing 10 ml of various solvents or purified water. The pH of the suspension was then adjusted with 2 M HCl or 0.1 M NaOH, if necessary. The suspension was continuously mixed at ambient temperature. An aliquot of the sample (5 ml) was taken at 24 and 48 h intervals. Each withdrawn sample was filtered using a 0.45 μm PTFE filter. The first 2 ml of the filtrate was collected for pH measurement. Additional 2 ml of the remaining filtrate was analyzed directly by HPLC as described previously.

2.3. Liposome preparation

LE-SN38 was prepared by the modified thin-film hydration method. Briefly, the hydrophobic excipients, such as lipids (phosphatidylcholine, cholesterol and cardiolipin) and α-tocopherol were dissolved in ethanol and were transferred into a suitable round bottom flask. The flask was then connected to a Büchi

R205 rotary evaporator (Flawil, Switzerland) and water bath (Buchi B-490) with temperature maintained at 35–40 °C. Vacuum was applied to the flask to evaporate the ethanol and form a homogeneous lipid film on the flask wall. The dry lipid film was maintained overnight under vacuum to remove traces of ethanol. The lipid film was then hydrated with a solution containing SN-38 dissolved in alkaline pH and 10–20% sucrose by rotating the flask at about 200 rpm at ∼40 °C until the lipid film was completely hydrated and a homogeneous dispersion was formed (approximately 1 h). The liposome dispersion was then extruded under a nitrogen atmosphere through two stacked of 0.2 and 0.1 μm polycarbonate filters (Whatman, Inc., Clifton, NJ, USA) using a high-pressure extruder (NeoPharm, Inc.) at room temperature. The mean vesicle size of liposomes was reduced to about 150 ± 20 nm. The resulting liposome-based drug formulation was then sterile filtered through 0.2 μm filter and filled into 10-ml serum vials. The filtered formulation in vials was lyophilized using VirTis lyophilizer (Genesis, The VirTis Company, Gardiner, NY, USA). The lyophilization cycle consisted of cooling the solution down to −45 °C for 2 h, primary drying for 24 h at −30 °C under 150 mTorr chamber pressure, ramp from −30 to −5 °C for 24 h under 150 mTorr chamber pressure, and second drying at 25 °C for 8 h under 200 mTorr chamber pressure.

2.4. Characterization of liposomes

2.4.1. Vesicle size measurement

Immediately after preparation, LE-SN38 was examined for possible aggregation by visual inspection. Thereafter, the liposome mean diameter and particle size distribution were determined using dynamic light scattering (DLS) technique with a Nicomp 380 Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA) equipped with auto dilution function. The laser in this equipment was operated at 632.8 nm using a 90° angle between incident and scattered beams. Polystyrene bead standards were used to verify the performance of the instrument prior to sample measurement. Data were analyzed automatically either by Gaussian Analysis (the least-squares quadratic: cumulants) or Nicomp Distribution Analysis (inversion of the Laplace transform) from the Nicomp CW388 software. Data were analyzed in terms of intensity, volume and number distributions assuming that the particles are spheres of uniform density which scatter light according to classical Mie Theory. Data were reported as volume weighted distribution and represented as mean of at least two measurements.

2.4.2. Freeze-fracture electron microscopy

Liposome morphologic analysis was carried out by the freeze-fracture electron microscopy. Samples were quenched using sandwich technique and liquid nitrogen-cooled propane (Sternberg, 1993). During the experiment, a cooling rate of 10,000 K/s was reached to avoid ice crystal formation and artifacts possibly caused by the cryo-fixation process. The fracturing process was carried out in JEOL JED-9000 freeze-etching equipment (JEOL, Ltd., Tokyo, Japan), and the exposed fracture planes were shadowed with platinum for 30 s at an angle of 25–35° and with carbon for 35 s (2 kV/60–70 mA, 1×10^{-5} Torr). The platinum replicas produced this way were cleaned with concentrated, fuming HNO_3 for 24 to 36 h followed by a repeated agitation with fresh chloroform/methanol (1:1, v/v) at least five times. Subsequently, these cleaned replicas were examined with a JEOL 100 CX (JEOL, Ltd., Tokyo, Japan) electron microscope.

2.4.3. Drug entrapment efficiency

Drug entrapment efficiency was determined by ultra-centrifugation method. An aliquot of the reconstituted LE-SN38 was diluted four-fold with normal saline and then centrifuged at 200,000 × *g* for 2 h at 4 °C using a Beckman Optima™ Series L-90K ultracentrifuge (Palo Alto, CA, USA) with a SW 60 Ti rotor. The total drug concentrations in liposomes before centrifugation and in supernatant after centrifugation were determined by HPLC as described under analytical methods section. The percentage of the drug entrapped in the liposomes was calculated as follows:

$$\text{Percent drug entrapment} = \frac{\text{drug}_{\text{total}} - \text{drug}_{\text{supernatant}}}{\text{drug}_{\text{total}}} \times 100$$

2.4.4. In vitro drug release

In vitro release of SN-38 from liposomal formulation was analyzed by membrane dialysis against

phosphate-buffered saline (PBS, pH 7.4) at 37 °C. Briefly, a 2 ml aliquot of reconstituted LE-SN-38 sample was placed in the dialysis cassette (Pierce Slide-A-Lyzer® dialysis cassettes with a MW cutoff of 10 K) and then suspended in a temperature-controlled, jacketed flask containing 400 ml of PBS. At various time intervals, aliquot samples were withdrawn and assayed for SN-38 content by HPLC method described previously. Drug release profiles (percent release versus time) were plotted.

2.5. Stability study

The stability of the lyophilized, reconstituted and diluted LE-SN38 were evaluated after storage at 2–8 and 20–25 °C (RT) for an extended period of time. The pH, lipid content, drug concentration, particle size distribution and drug entrapment efficiency of the samples were determined as a function of the storage time. The drug and lipid content in the formulation were determined by HPLC methods described previously. The vesicle size and distribution of the liposome-based SN-38 was determined using the Nicomp sub-micron particle sizer.

2.6. In vitro cytotoxicity study of LE-SN38

In vitro cytotoxicity of LE-SN38 and CPT-11 in cancer cell lines was determined using Sulforhodamine B (SRB) assay (Monks et al., 1991). A total of eight cancer cell lines, including human colon cancer (HT29), human lung cancer (A549), human breast cancer (MX-1), human ovarian cancer (OVCAR-3), human pancreatic cancer (Capan-1), mouse Leukemia (P388), mouse adriamycin-resistant leukemia (P388/ADR) and Lewis lung carcinoma (LLC), were included in this study. The GI_{50} value was calculated as the concentration of LE-SN38 or CPT-11 that gives 50% growth inhibition (Skehan et al., 1990).

2.7. Multiple dose toxicity study of LE-SN-38 in CD2F1 mice

CD2F1 mice (male and female) were obtained through Harlen Sprague Dawley Laboratories (Indianapolis, IN). The average weight of mice on day 1 of study was 16–22 g for females and 20–27 g for males with an age of 6–7 weeks. Mice were pre-weighed individually prior to experiment. On days 1 through 5, animals were injected intravenously via tail vein with LE-SN38 or placebo liposomes at 5, 7.5 and 10 mg/kg dose levels. All animals were observed once daily during the study periods for mortality and clinical signs. Animals showing toxicity as manifested by clinical signs and body weight loss of 25% or more were considered as moribund and euthanized immediately.

2.8. Therapeutic efficacy of LE-SN38 and CPT-11 in xenograft mouse tumor models

Either female CD2F1 (6–8 weeks old) mice or female CB-17 SCID mice (4–6 weeks old) were obtained from the vendor and maintained as described previously. The CD2F1 mice were transplanted with P388 murine leukemia tumor cells, whereas the SCID mice were transplanted with HT-29 human colon cancer cells, Capan-1 human pancreatic cancer cells and MX-1 human breast cancer cells. After a suitable waiting period (waiting period varied based on the tumor models), each mouse received intravenous injection via tail vein of placebo liposomes, LE-SN-38 or CPT-11 at pre-determined dose levels. For P388 bearing mice, the long-term survival for each treatment group was assessed, whereas for solid tumor bearing mice, the tumor growth inhibition of placebo liposomes, LE-SN-38 or CPT-11 at different dose levels was measured after 28-day post-treatment.

3. Results and discussion

3.1. Preformulation

Preformulation studies were conducted to investigate the solubility of SN-38 in various solvents and pH-dependent equilibrium of active lactone ring and inactive carboxylate form of SN-38. The results of the solubility study are summarized in Table 1. It is shown that the solubility of SN-38 in water, pH 3.5 and 7.4 buffers was estimated as 11–38, 7.2 and 36 μg/ml, respectively, which suggest that these solvents cannot effectively solubilize the drug. SN-38 exhibited a fairly good solubility (>5 mg/ml) in some of the organic sol-

Table 1
Solubility of SN-38 in different solvent systems

Solvent system	Estimated solubility
Water	11–38 μg/ml (clear and colorless)
pH 3.5 acetate buffer or lactate buffer	~7.2 μg/ml
pH 7.4 phosphate-buffered saline	~36 μg/ml
0.1 M NaOH	>4 mg/ml (yellow)
DMSO	>10 mg/ml
1-Methyl-2-piperidone	>7.4 mg/ml
Water/1-methyl-2-piperidone (1:1)	<1 mg/ml
2-Hydroxypropyl-beta-cyclodextrin (drug:cyclodetrin = 1:2 and 1:1)	<1 mg/ml
Water/methylene chloride (1:1, v/v), pH 8.0	1.77 mg/ml
Water/methylene chloride (1:1, v/v), pH 8.5	1.56 mg/ml

vents investigated; however, these solvents are not acceptable for use in pharmaceutical products. It was found that the solubility of SN-38 increased significantly in alkaline solution at pH 9 and above, which indicates that the solubility of open carboxylate form of SN-38 is much higher than that of closed lactone form of SN-38.

The conversion of SN-38 (lactone) to carboxylate form under different pH conditions was investigated by diluting SN-38 stock solution in acetonitrile with 50/50 (v/v) acetonitrile/buffers at different pH conditions. After the SN-38 sample equilibrated to a specific pH, it was analyzed for SN-38 lactone form and SN-38 carboxylate by HPLC. SN-38 and SN-38 carboxylate can be well baseline separated by the HPLC method. As shown in Fig. 3, approximately 60% of SN-38 was converted to the SN-38 carboxylate at neutral pH (pH 7.1). At pH 10.2, greater than 95% of SN-38 was converted to the SN-38 carboxylate. When pH was lowered to 3.4, approximately 100% of SN-38 remained in the closed lactone ring form. This finding confirmed the results of an earlier study conducted by Gelderblom et al. (1999). Additionally, it was found that 100% of SN-38 carboxylate was converted back to the SN-38 lactone form when pH was adjusted to 3. It was also discovered that the color of SN-38 solution changed from clear and colorless to bright yellow when pH of the solution was adjusted from 3 to about 9. The color change and structural conversion of SN-38 to the carboxylate form were reversible and dependent on the pH of the medium. The results of pH-dependent solubility and pH-dependent equilibrium behavior of SN-38 formed the basis of the novel approach to formulate a stable, parenteral liposome-based SN-38.

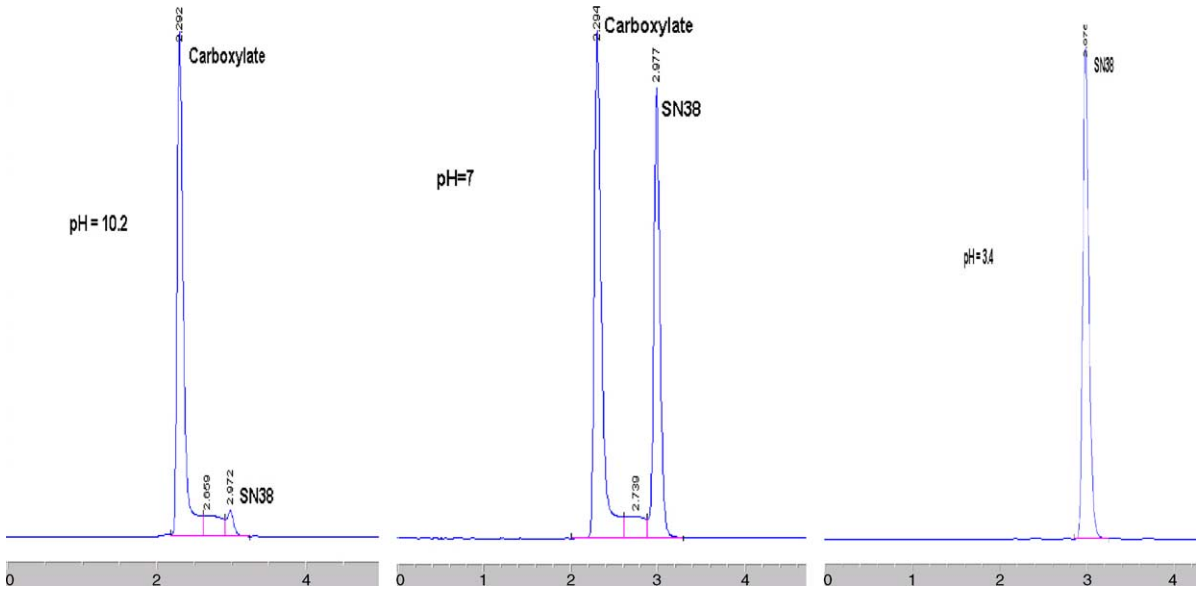

Fig. 3. HPLC chromatograms of SN-38 at different pH medium.

3.2. Formulation development

The goals of the formulation development were to overcome solubility problems of SN-38, achieve high drug entrapment in the liposomes and improve stability of LE-SN38. Based on the preformulation data, the novel formulation approach was to prepare liposome-based SN38 at an alkaline pH (~10), in which SN38 was soluble and in its inactive carboxylate form, and then the liposomal SN-38 was lyophilized. After lyophilization, the pH of the reconstituted LE-SN38 was lowered to less than 3 in order to facilitate conversion of the carboxylate form to active SN-38 and entrap the drug molecules inside the liposome bilayer.

Several LE-SN38 prototype formulations were investigated in terms of lipid to drug ratio, drug concentration, drug entrapment, filterability and physical and chemical stability. Upon further optimization, a lead formulation of liposome-based SN-38 was developed. The lead formulation consisted of 2 mg/ml of SN-38, DOPC, cholesterol and cardiolipin. The drug-to-lipids ratio and DOPC: cholesterol: cardiolipin mole percent ratios were 1:18 and 50:40:10, respectively. Since lipid components in the liposomes are heat labile, steam sterilization of liposomes was not a viable approach. To achieve sterile filterable liposome formulation, LE-SN-38 was size reduced to about 150 nm by extrusion to allow for aseptic filtration through 0.22 μm membrane filter (PVDF membrane filter). There was neither appreciable loss (<1%) of drug and lipids nor a change in liposome vesicle size distribution post-filtration.

To enhance the chemical and physical stability of the liposome formulation, freeze-drying or lyophilization was used to remove free water from the formulation to minimize lipid hydrolysis. Phospholipids in liposomes are known to be sensitive to hydrolysis and oxidation in aqueous medium. Liposomes can be hydrolyzed to form lysophospholipids and free fatty acids. The lysophospholipids can be further hydrolyzed to glycerophospho compounds and fatty acids (Kensil and Dennis, 1981; Grit et al., 1989, 1993a,b; Zhang and Pawelchak, 2000). The hydrolytic degradation may change the rigidity of liposomal bilayers, retention of entrapped drug, and alter liposome size and distribution. Lyophilization would be expected to protect the liposome components from hydrolysis as it removes free water from the product; however, the preservation of the structural integrity of liposome during dehydration/rehydration process has presented considerable challenges to the pharmaceutical scientists. In the absence of any protective agents, vesicle fusion and leakage of internal aqueous contents of liposomes can occur (Harrigan et al., 1990). Sugars have been shown to act as protective agents during dehydration/rehydration of liposomes to prevent vesicle fusion and retention of encapsulated compounds within liposomes (Madden et al., 1985; Crowe et al., 1985). The ability of sugars, such as trehalose and sucrose, to prevent vesicle fusion was evaluated by measuring the vesicle size and drug entrapment efficiency after the formulation was lyophilized and reconstituted. It was found that both trehalose and sucrose were equally effective in protecting LE-SN38 during lyophilization. The optimum sugar-to-lipid molar ratio found to be effective in protecting LE-SN38 liposomes ranged from 3:1 to 6:1.

Color change (from bright yellow to milky white) was observed immediately after lyophilized LE-SN38 was reconstituted with the acidic buffer, such as lactate buffer, indicating the structural conversation of SN-38 carboxylate to a more hydrophobic and lipophilic SN-38. Upon conversion, SN-38 partitioned into the liposome bilayer. In addition, during rehydration/reconstitution, liposomes and drug molecules restructured to entrap more 'free' drug into the liposome bilayer. Furthermore, after rehydration, SN-38 in liposomes would have a concentration gradient across liposome membranes. Fluorescence measurements have indicated that SN-38 is located in the outer leaflet of the liposome bilayer (close to the water phase, Peikov et al., unpublished results). The drug loading mechanism as depicted in Fig. 4 requires the structural conversion of SN-38 under acidic condition.

3.3. Liposome vesicle size of LE-SN38

The freeze-fracture electron microscopy images of reconstituted and pre-lyophilization of LE-SN38 are presented in Fig. 5. The liposome vesicles as shown under electron microscope are discrete particles with sharp boundaries that range in size from 150 to 200 nm, which matches the results obtained from particle size measurements using DLS technique. After lyophilization and reconstitution with acidic

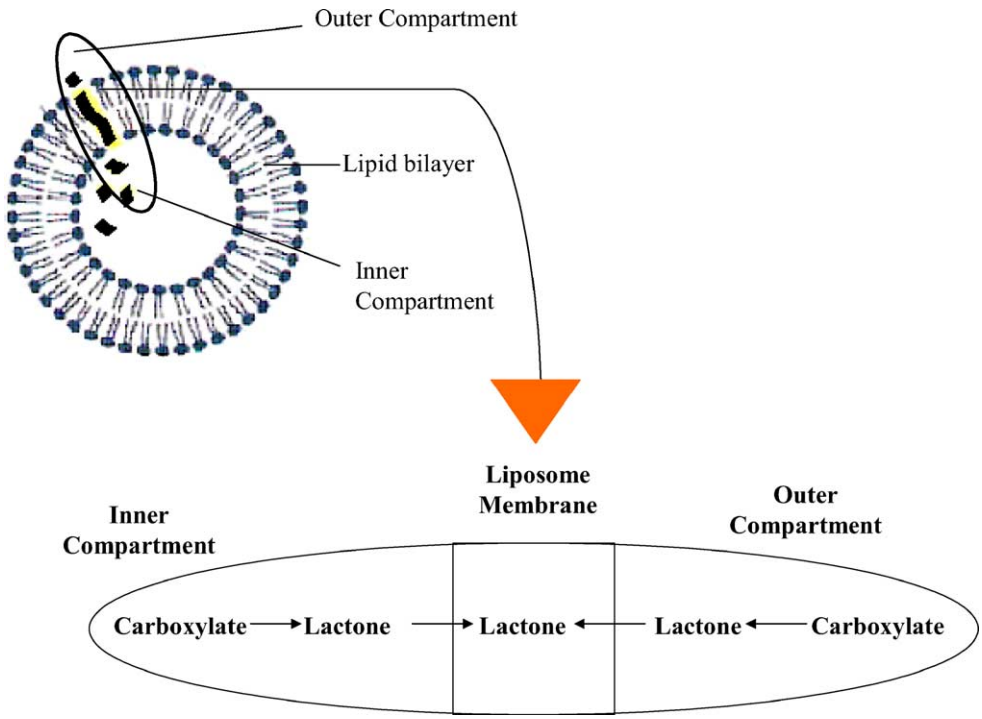

Fig. 4. A schematic representation of SN-38 drug loading at acidic pH condition at alkaline condition, SN-38 exists as hydrophilic carboxylate forms inside and outside of the liposomes. At acidic condition, SN-38 converts to the hydrophobic closed lactone forms and moves into the liposome bilayer.

buffer, the mean liposome vesicle size increased by about 15% as compared to the mean diameter of the pre-lyophilization samples. The vesicle size distribution of the samples under both conditions was mono-model (Gaussian) distribution. The swelling of the liposomes post-reconstitution may be due to the drug being activated (to form the closed lactone ring structure) and partitioned into the liposome bilayer as the drug entrapment efficiency increased from about 8% for pre-lyophilized sample to greater than 95% for post-lyophilized and reconstituted sample.

3.4. *In vitro drug release*

Drug release profile of LE-SN38 is shown in Fig. 6. It was found that there was approximately 1.9% drug release from LE-SN-38 into PBS buffer over 120 h, which suggests that the SN-38 remained associated with the liposomes in the formulation during the course of study. It also suggests that LE-SN38 would be stable during clinical use when diluted with either normal saline or PBS buffer. The drug release data obtained by dialysis confirmed the drug entrapment efficiency results determined by ultra-centrifugation method (>95%).

3.5. *Short- and long-term stability*

LE-SN38 was physically and chemically stable at 2–8 °C for up to 6 months (Table 2). No significant changes in mean vesicle size, pH and drug entrapment were observed during the course of stability study. Eight-fold diluted LE-SN38 stored at 2–8 and 25 °C was physically and chemically stable for up to 8 h (Table 3). Initially, the mean vesicle diameter was 173.5 nm. At the 8-h time point, the mean vesicle diameter remained relatively constant at both storage temperatures. No precipitation or drug crystals were observed. SN-38 concentration remained unchanged at both temperature conditions over the course of the

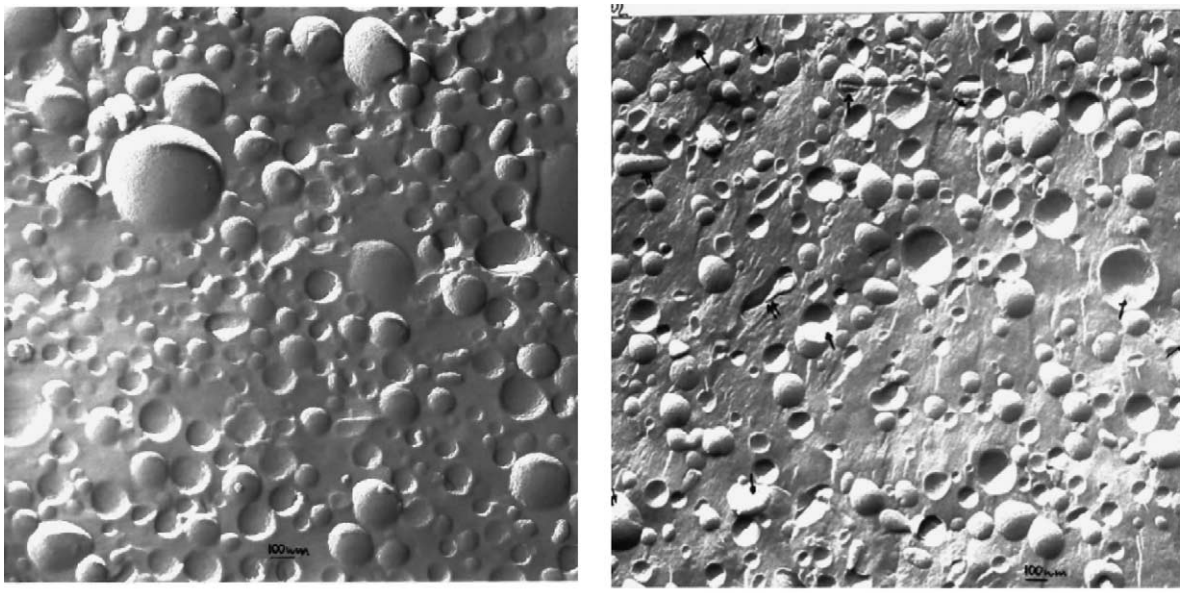

Fig. 5. Freeze-fracture electron micrographs of (a) reconstituted LE-SN38, (b) liquid formulation of LE-SN38 before lyophilization. The mean liposome vesicle size of (a) an (b) is 139 and 121 nm, respectively as measured by DLS.

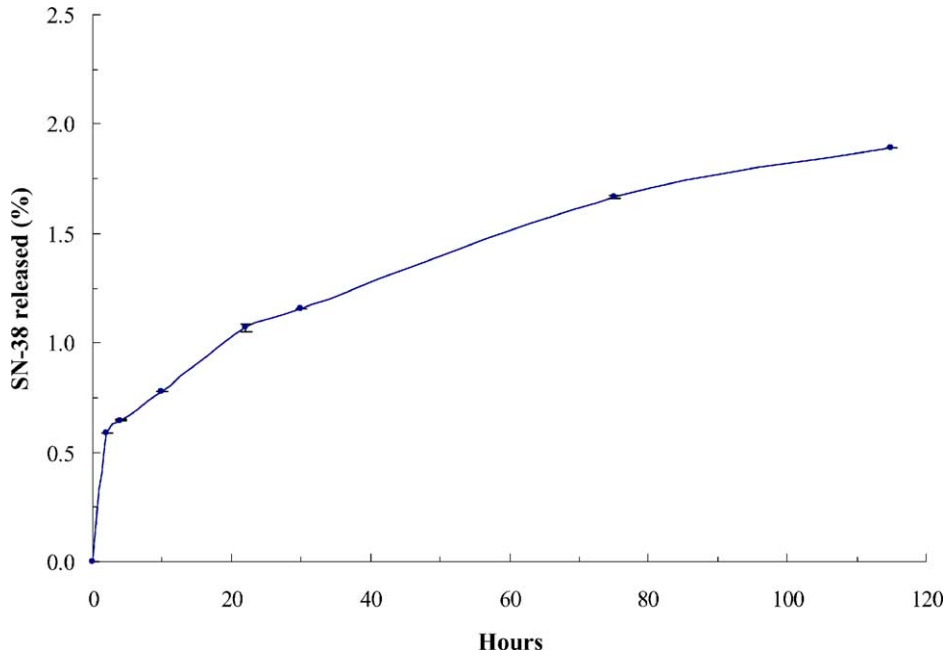

Fig. 6. SN-38 release profile from LE-SN38.

Table 2
Stability data of lyophilized LE-SN38 stored at 2–8 °C[a,b]

Time (month)	SN-38 percent of initial	Cardiolipin percent of initial	Cholesterol percent of initial	DOPC percent of initial	pH	Appearance	Entrapment efficiency (%)	Mean Vesicle size (nm)
Initial	100	100	100	100	2.28	Yellow lyo cake	>95	154
1	103	100	101	97	2.28	Yellow lyo cake	>95	172
3	96.5	92.3	102	100	2.27	Yellow lyo cake	>95	178
6	98.4	90	105	94.5	2.10	Yellow lyo cake	>95	139

[a] All values are mean values of duplicate samples for each time point.
[b] Lipid and drug results are percent of the initial concentrations as measured at time zero.

stability study (Table 3). The entrapment efficiency remained unchanged regardless of the storage time and condition. DOPC, cholesterol and cardiolipin concentrations decreased slightly from initial values at 8 h post dilution. There was no appreciable change in pH for the diluted samples over the 8-h study period at both temperatures (Table 3).

3.6. In vitro cytotoxicity

Study showed that all eight cell lines investigated were sensitive to LE-SN38 with GI_{50} values less than 0.1 μM. These in vitro cytotoxicity results were comparable to the data of free SN-38 dissolved in DMSO that were previously reported (Lavelle et al., 1996; Cavaletti et al., 2000) indicating SN-38 was released from the liposomes during incubation of LE-SN38 in cell cultures and was available for inhibiting cell growth. The results indicated that LE-SN38 was approximately 200–2000-fold more cytotoxic than CPT-11 against all tumor cell lines (Chien et al., 2003).

3.7. Multiple dose toxicity study of LE-SN38 in CD2F1 mice

The results of the multiple-dose toxicity study of LE-SN-38 in CD2F1 mice indicated that the average weight loss ranged from 5.2% for the 5 and 7.5 mg/kg dose groups (5 and 7.5 mg/kg × 5 days) and 15.7% for the 10 mg/kg dose group (10 mg/kg × 5 days). However, the weight lost was recovered by day 17 post-treatment for all LE-SN38 treatment groups. Animals in all groups were acting normal on days 1–5 post-injection of LE-SN38. On days 6–12, animals treated with 5 and 7.5 mg/kg for 5 days were also normal, whereas animals treated with 10 mg/kg for 5 days showed clinical symptoms manifested by hunched posture, rough coat, dehydration and decreased activity. On days 14–18 post-injection, all animals from all groups recovered (Sarkar et al., 2003). In general, LE-SN38 was well tolerated in mice at all dose levels studied. This observation could be attributed to the use of non-toxic lipids to form liposomes that buffered the toxicity of SN-38. The retention of the

Table 3
Stability data for eight-fold diluted reconstituted samples at 2–8 and 25 °C[a,b,c]

Time (h)/ storage condition		SN-38 percent of initial	Cardiolipin percent of initial	Cholesterol percent of initial	DOPC percent of initial	pH	Appearance	SN-38 entrapment (%)	Mean vesicle size (nm)
0	N/A	100	100	100	100	2.98	Off-white, translucent	>95	174
8	2–8 °C	100	100	93.3	95.7	2.98	Off-white, translucent	>95	176
8	25 °C	99.2	96.0	93.3	95.7	2.95	Off-white, translucent	>95	183

N/A: not applicable.
[a] The reconstituted LE-SN38 was diluted eight-fold with normal saline.
[b] All values are mean values of duplicate samples for each time point.
[c] Lipid and drug results are percent of the initial concentrations as measured at time zero.

drug in the liposomes could have reduced the tendency of SN-38 molecules to directly interact with normal cells, thereby attenuating the overall toxicity related to free SN-38.

3.8. Therapeutic efficacy of LE-SN38 and CPT-11 in xenograft mouse tumor models

The therapeutic efficacy of LE-SN38 and CPT-11 against different tumors in mice was summarized in Table 4. For P388 tumor bearing mice administered with CPT-11 at doses of 4 and 8 mg/kg for 5 consecutive days, the median survival time was 16 and 20 days, respectively, with no long-term survival. A median survival time of 22 days and 22% long-term survival (up to 60 days) was observed for the mice administered with 16 mg/kg CPT-11. In contrast, when mice were given LE-SN38 at doses of 2.76 and 5.52 mg/kg for 5 consecutive days, 60 and 100% long-term survival (up to 60 days) was observed at the respective dose levels. There were no clinical signs of toxicity, such as diarrhea, hunched posture, scruffy fur and alopecia or weight loss at these dose levels of LE-SN-38. Evidently, LE-SN38 exhibited significantly greater therapeutic efficacy against P388 murine leukemia tumor than the prodrug CPT-11 (Kamath et al., 2003).

When LE-SN38 was given to mice bearing HT-29 human colon tumor at dose 2, 4 and 8 mg/kg, LE-SN38 inhibited human colon cancer growth by 46, 70, and 88%, respectively, at 28 days post-treatment. In contrast, for mice treated with CPT-11 at the same dose levels, only 36% inhibition was observed at the highest dose level (8 mg/kg). At 2 and 4 mg/kg dose levels, CPT-11 did not show any inhibition against tumor growth. Clearly, LE-SN38 exhibited much greater inhibition against HT-29-induced tumor in mice than the prodrug CPT-11 at the same dose levels (Lei et al., unpublished results).

In addition, LE-SN38 exhibited greater activity against Capan-1 human pancreatic tumor growth in the animal groups treated with LE-SN38 compared to those treated with CPT-11 (Table 4). It was demonstrated that the anti-tumor efficacy of LE-SN38 against human pancreatic tumor in the SCID ectopic model was superior to CPT-11 (Pal et al., 2003). Moreover, it was found that LE-SN38 induced a dose-dependent tumor regression of MX-1 human breast solid tumor in SCID mice. For mice treated with LE-SN38 at 4 and 8 mg/kg dose levels, the tumor regressed by 43.9

Table 4
Anticancer effects of LE-SN38 or CPT-11 against various tumor models in mice

Treatment	Tumor models			
	P388 murine leukemia 60-day percent survival ($n = 10$)	HT-29 human colon percent growth inhibition[a] ($n = 5$)	Capan-1 human pancreatic percent growth inhibition[a] ($n = 5$)	MX-1 human breast percent growth inhibition[a] ($n = 5$)
Placebo liposomes	−	→	→	→
CPT-11				
2 mg/kg × 5	n/t	→	n/t	n/t
4 mg/kg × 5	−	→	n/t	n/t
8 mg/kg × 5	−	↑	↑↑	→
16 mg/kg × 5	+	n/t	↑↑↑	n/t
LE-SN-38				
2 mg/kg × 5	+++ (2.78 mg/kg × 5)	↑↑	n/t	n/t
4 mg/kg × 5	+++++ (5.52 mg/kg × 5)	↑↑	↑↑↑	↑↑
8 mg/kg × 5	n/t	↑↑↑↑	↑↑↑↑↑	↑↑↑↑
12 mg/kg × 5	n/t	n/t	↑↑↑↑↑	n/t

n/t: not tested. The number of mice used in each treatment for the studies ranged from 5 to 10. →: no inhibition; ↑: increase in inhibition by more than 20%; +: increase in percent survival by more than 20%; −: no long-term survival.

[a] Percent growth inhibition is defined as the percentage of final tumor volume as compared to the initial tumor volume. It is calculated using the following formula: Percent growth inhibition $= (V_t / V_{initial}) \times 100$. The drug treatment was initiated when the tumor reached to a size of 65–120 mm^3. The final tumor was measured on day 28 post-treatment.

and 87.8%, respectively. However, for mice given CPT-11 at 8 mg/kg dose level, there was no significant reduction of tumor size (Lei et al., unpublished results).

It is known that intravenous administration of liposomes will lead to their accumulation in extravascular sites that exhibit leaky vasculature, as in the case for tumor sites. For LE-SN38, the extent of this accumulation could lead to an increase in tissue-specific delivery of SN-38 at a drug level corresponding to several orders of magnitude greater than its precursor, CPT-11. This passive delivery of drug to the therapeutic targets may account for the greater efficacy of SN-38 versus CPT-11. Liposomes may also act to protect SN-38 from structural transformation and/or chemical degradation. This protection of the active molecule could have led to a significant increase in bioavailability, which ultimately enhanced the drug potency and efficacy. In summary, the anti-tumor efficacy of LE-SN38 was much greater than that of CPT-11 at the same dose levels.

Camptothecin analogues have shown significant anti-tumor activity in mice bearing various tumors. They have been investigated extensively at pre-clinical and clinical developmental stages. However, the clinical use of the camptothecin drugs has been limited by their undesirable chemical properties, such as poor water solubility and instability at high pH condition, and high toxicity as described previously. Previous investigators have attempted to develop liposomal formulations of camptothecin drugs to enhance drug solubility, stability, and attenuate toxicity. Cortesi et al. studied the liposomal camptothecin (CPT) formulations containing: egg phospholipids (Egg PC) and cholesterol; Egg PC, cholesterol and didodecyl-dimethyl-ammonium bromide (DDAB, cationic lipid); Egg PC, cholesterol and dicetyl-phosphate (DCP, anionic lipid); and found that the liposomal CPT showed similar or enhanced anti-proliferative activity against cultured human leukemic K562 cells (Cortesi et al., 1997) compared to the free CPT. However, the drug entrapment was only in the range of 32–57%. Burke and Mi (1993) has shown that the entrapment of camptothecins within lipid bilayer conferred protection of the lactone ring structure of camptothecin drugs against hydrolysis. This approach, however, is limited by the very low drug concentration that can be entrapped into liposome membranes. In the present study, we have developed a novel liposome-based SN-38 that consists of a clinical relevant drug concentration and high drug entrapment. The product has exhibited 200–2000-fold greater cytotoxicity and improved anti-tumor therapeutic efficacy over CPT-11 at the same dose levels. A systematic approach was undertaken to develop and optimize the formulation. It involved formulation of SN-38 in its inactive form, lyophilization to achieve acceptable shelf-life, and then re-conversion of the inactive form of SN-38 to active form of SN-38 prior to administration to patients. The formulation process is novel and pharmaceutically scalable. The formulation and preclinical findings of this novel liposome-based SN-38 warrant further clinical development of this product.

4. Conclusion

LE-SN38, a stable sub-micron liposome-based formulation, has been developed for clinical use. It was formulated utilizing a novel approach which involved the solublization of SN-38 by opening the lactone ring in alkaline medium to yield an acceptable and clinically relevant concentration. The formulation was then filter-sterilized and lyophilized to achieve acceptable shelf-life. Subsequently, the open form of SN-38 in liposomes was activated in acidic buffer prior to administration to the patient. LE-SN38 was formulated with the biocompatible excipients that have been used in the clinical trials for several NeoPharm's products. In addition, LE-SN38 allows delivery of active SN-38 directly to the specific site of action and obviates the need for enzymatic conversion; therefore, it may be clinically more efficacious than its prodrug, Camptosar®, CPT-11 at the same therapeutic dose. Finally, LE-SN38 exhibited a broader spectrum of anti-tumor activities as compared to CPT-11 against several tumor cell lines in pre-clinical studies. Phase I clinical trials of LE-SN38 are currently in progress.

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